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Cloning and expression of *cry*1*Ac*20 gene from *Bacillus thuringiensis* strain Rpp02

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Abstract：*Bacillus thuringiensis* subsp. *morrisoni* strain Rpp02 isolated by researchers in our laboratory was highly toxic to several kinds of insect pests. Results of PCR analysis indicated that strain Rpp02 contained the *cry*1*Ac* gene，and subsequently a novel *cry*1*Ac* gene was cloned from this strain by PCR techniques. The results of sequence analysis showed that the novel gene *cry*1*Ac*20，named by *B*. *thuringiensis* Pesticidal Crystal Protein Nomenclature Committee，contained an open reading frame of 3 534 nucleotides encoding a protein of 1 177 amino acids with a predicted molecular mass of 133.144 kDa and isoelectric point of 4.952. Compared with other known *cry*1*Ac* genes，*cry*1*Ac*20 has shown as high as 99% nucleotide sequence homology. Bioassay showed that the toxic protein appeared high insecticidal activity against *Pieris rapae* L.，and the corrected mortality was 88.78% 48 h after feeding with leaves immersed in cultures containing transformants.

Key words：*Bacillus thuringiensis*；*cry*1*Ac*20 gene；cloning；expression

Bacillus thuringiensis is a rod-shaped，ubiquitous gram-positive bacterium. During sporulation，it produces large crystalline parasporal inclusions (*cry* proteins)，many of which are highly toxic to many insects belong to Lepidoptera，Diptera，Coleoptera，and Hymenoptera as well (Hofte and Whiteley，1989；Schnepf *et al*.，1998). Since the first cloning of *cry* gene from strain HD-1Dipel by Schnepf in 1981 (Schnepf *et al*.，1981)，there were over 320 kinds of such protein genes had been isolated and cloned. These genes，according to the similarities of their amino acid residues，can be classified into 49 groups，93 subgroups，147 classes and 328 subclasses (Crickmore *et al*.，1998). Among them，*cry*1 is a group of relatively highly homologous genes which exhibit highly specific toxin to Lepidoptera，while *cry* protein encoded by *cry*1*Ac* gene is high toxic to *Plusia ni*，*Heliothis virescens*，and *Helicoverpa armigera* of Lepidoptera (Palidam，1992).

*Cry*1*Ac* gene was firstly cloned by Adang *et al*. from *B*. *thuringiensis* subsp. *kurstakai* (Adang *et al*.，1985). Up to now，there have been 19 genes of such subclass that were cloned and sequenced in succession in the world，and most of them were originated from different strains of *B*. *thuringiensis* subsp. *kurstakai* (Schnepf *et al*.，1998；http://www.biols.susx.ac.uk/Home/Neil-Crickmore/Bt/). Sun *et al*. (1996) cloned a *cry*1*Ac* gene registered as *cry*1*Ac*10 in GenBank in 1997，while Yao *et al*. (2003) cloned another *cry*1*Ac* designated as *cry*1*Ac*14 from strain Ly30. *Cry*1 genes have been widely used for insect resistant transgenic plants. Monsanto Company had successfully introduced *cry*1*Ac* gene into cotton lines，resulting in new varieties resistant to insects，which cover over 50% of total cotton planting areas due to their high resistance against *Helicoverpa armigera*；Guo *et al*. (1996) had also successfully introduced artificially synthetic *cry*1*Ac* gene into cotton planting varieties for controlling *H*. *armigera*. For years，development and research on highly toxic and new insecticidal *cry* genes of *B*. *thuringiensis* has long been one of the popular spots in areas of basic research and utilization at home and abroad.

Strain Rpp02，isolated by researchers in our laboratory from Sichuan ecological condition，is a kind of *B*. *thuringiensis* strain highly toxic to Lepidoptera insects. This strain belongs to *B*. *thuringiensis* subsp. *morrisoni* through identification. In this study，the

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strain Rpp02 was used as the source for cloning of *cry*1*Ac* gene. The whole-length *cry*1*Ac* gene was cloned from this strain and expressed in *Escherichia coli* to verify its insecticidal activities. This gene may provide useful genetic resource for constructing insecticidal engineering bacteria and cultivating transgenic plants resistant against insects.

1 MATERIALS AND METHODS

1.1 Strains and plasmids

Bacteria strains and plasmids used in this study were listed in Table 1.

Table 1 Strains and plasmids used and constructed in this study

Strains and plasmids	Characterization	Source
Strains		
Bt Rpp02	*B. thuringiensis* subsp. *morrisoni* wild type	Stored in PPBCL
E. coli DH5α	$supE44\ \Delta lacU_{169}$（Ø80 $lacZ\Delta M15$） has $R17$ $recA1$ and $A1\ gyr\ A_{96}\ thi^{-1}\ relA1$	Stored in PPBCL
DHF10	*E. coli* DH5α containing pGF10	This study
Plasmids		
pGEM-T vector	Amp^R	Stored in PPBCL
pGF10	pGEM-T vector carried 3 734 bps of *cry*1*Ac*	This study

PPBCL：Plant Protection and Biological Control Laboratory，Rice Research Institute，Sichuan Agricultural University，Wenjiang，Sichuan.

1.2 Media

E. coli and *B. thuringiensis* were grown on Luria-Bertani media containing 1% tryptone，0.5% yeast extract，and 1% NaCl（Sambrook *et al*.，2002）；the pH value of the medium had been adjusted to 7.0. Selective media contained 60 μg/mL of ampicillin，100 μg/mL of isopropyl-β-D-thiogalactopyranosid（IPTG）and 50 μg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside（X-gal）.

1.3 DNA manipulation

The extraction of plasmids，DNA fragment recovery and other DNA manipulations were carried out according to reference（Sambrook *et al*.，2002）.

1.4 Cloning and sequencing of *cry*1*Ac* gene

According to the whole length of *cry*1*Ac* gene published on GenBank，a pair of primers（P1：5′ AACCTGAGTTTGCATGAGAC3′；P2：5′ ATAATGGG CGAGAAGTAAGT3′）had been designed and synthesized. The PCR reaction system was based on Sambrook *et al*.（2002）.

The purified PCR products were recovered，ligated to pGEM-T vector and transferred to *E. coli* DH5α competent cells. Positive clones were screened by alpha-complementation，and at last PCR identification was performed（Sambrook *et al*.，2002）. The sequencing was done by Shanghai Sangon Biological Engineering & Technology and Service Co. Ltd.

1.5 Expression of *cry*1*Ac* gene in *E. coli*

The transformant *E. coli* strain DH5α was grown in liquid Luria-Bertani medium supplemented with 60 μg/mL of ampicillin at 37℃ with shaking. The cell suspension was then subjected to centrifuge to collect cultural products and obtain bacteria. The bacteria were re-suspended by adding 10 mmol/L of Tris-HCl（pH 8.0）. Cells were broken up by sonicating for 15 min，and precipitates were collected by centrifuging and dissolved with 1/10 volume of Na_2CO_3（50 mmol/L，pH 9.5）. And finally，the expressed products were determined with routine sodium dodecyl sulphate-polyacrylamide gel electrophoresis（SDS-PAGE）（Sambrook *et al*.，2002）.

1.6 Bioassay

The transformant *E. coli* strain DH5α had been shacked for another 3 days in liquid Luria-Bertani medium supplemented with 60 μg/mL of ampicillin at 37℃ with a speed of 200 r/min. Moderate cabbage leafs were selected，washed and dried，and exposed to ultraviolet radiation for 15 min. The leaves were cut into an area of 2 cm × 2 cm，dipped in bacteria liquids for 5 min，and put into disinfected culture vessels to dry them after discarding excessive liquids. Each vessel encompassed 4 leaves，and leaves dipped in liquid Luria-Bertani medium were treated to controls. Each treatment and control contained 30 healthy 2nd or 3rd instar larvae of *Pieris rapae* L.，with 3 repeats at room temperature. The death toll of these insects had been observed，and the mortality and corrected mortality has been caculated consequently. Corrected mortality =（Mortality of experimental group − Mortality of control group）/（100 − Mortality of control group）.

2 RESULTS

2.1 Cloning of the whole *cry*1*Ac* gene

A fragment of about 3.7 kb was obtained by amplifying genomic DNA of Rpp02 strain with primers P1 and P2（Fig. 1）. The fragment had been cloned into pGEM-T vector. Three positive clones were screened among transformants by PCR amplification，one of them had been named DHF10，and recombinant plasmid containing target fragment had been named pGF10 at the same time.

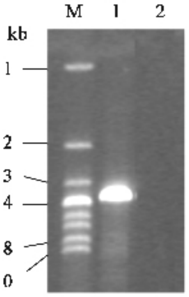

Fig. 1 PCR products amplified from strain Rpp02 with P1/P2 primers
M: 1 kb DNA marker; 1: PCR products; 2: Blank control.

2.2 Sequence analysis of *cry*1*Ac* gene

A nucleic acid sequence of 3 734 bp had been obtained by sequencing pGF10. The open reading frame of 3 534 nucleotides encoding a protein of 1 177 amino acids with a predicted molecular mass of 133.144 kD and isoelectric point of 4.952. This confirmed that it was a kind of weak acid protein, which contained 3 amino acids, Leu, Ser, and Glu with their highest proportions in content of 8.0%, 7.8%, and 7.7% respectively. The sequence of a site upstream of the coding regions, which can activate RNA polymerase, had been predicted via analysis of the full-length prediction software, Bacterial Sigma 7.0 Promoter, on Softberry website (Fig. 2). The sequence similarity of the gene with other reported ones was 99% with a few changes of several nucleotide and corresponding amino acids thereof (Table 2). According to Table 2 the most minor change occurred between *cry*1*Ac*5 and *cry*1*Ac*20 with only one different amino acid, namely Ile^{7}→Thr, while the greatest change occurred between *cry*1*Ac*1 and *cry*1*Ac*20 with 5 different amino acids, namely Ile^{7} → Thr, Phe^{148} → Leu, Leu^{366} → Phe, Phe^{440} → Ser, Asn^{442}→ deficiency, and Thr^{789} → Ala. However, the mutations of some sites did not bring about the change of amino acids because of the degeneracy of codons as illustrated in Table 2. This gene had been registered in GenBank with the accession number DQ285666 and named as *cry*1*Ac*20 by *B. thuringiensis* Pesticidal Crystal Protein Nomenclature Committee.

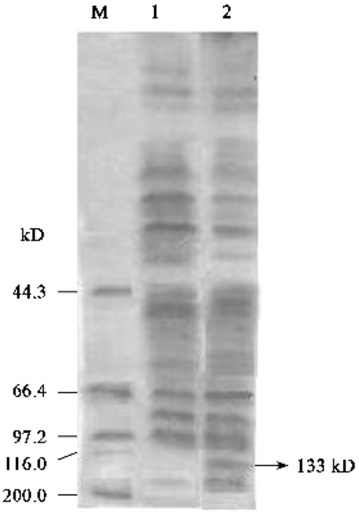

Fig. 2 Expressed *cry*1*Ac* in *E. coli* was analyzed by SDS-PAGE
M: Molecular weight marker; 1: *E. coli* DH5α without plasmid; 2: DHF10 with pGEM-F10.

Table 2 The difference of nucleotides between several *cry*1*Ac* genes

*cry*1*Ac* subclass	Amino acid position								
	7	148	206	366	440	442	655	789	802
*cry*1*Ac*20	acc	ttg	tat	ttt	tct	− − −	tat	gcc	tgc
	Thr	Leu	Tyr	Phe	Ser	−	Tyr	Ala	Cys
*cry*1*Ac*1	atc	ttt	tat	tta	ttt	aat	tat	gcc	tgc
	Ile	Phe	*	Leu	Phe	Asn	*	*	*
*cry*1*Ac*2	atc	ttg	cat	ttt	tct	− − −	tgc	gcc	ttc
	Ile	*	His	*	*	−	Cys	*	Phe
*cry*1*Ac*5	atc	ttg	tat	ttt	tct	− − −	tat	gcc	tgc
	Ile	*	*	*	*	−	*	*	*
*cry*1*Ac*10	atc	ttt	tat	tta	ttt	aat	tat	gcg	tgt
	Ile	Phe	*	Leu	Phe	Asn	*	*	*
*cry*1*Ac*11	atc	ttg	tat	tta	tct	aat	tat	gcc	tgc
	Ile	*	*	*	*	Asn	*	*	*
*cry*1*Ac*15	atc	ttt	tat	tta	tct	− − −	tat	gcc	tgc
	Ile	Phe	*	*	*	−	*	*	*
*cry*1*Ac*16*atc*	atc	ttt	tat	ata	ttt	aat	tat	acc	tgc
	Ile	Phe	*	Leu	Phe	Asn	*	Thr	*

Deleted DNA sequence was indicated by '− − −', deleted amino acid by '−', and the same amino acid by '*'.

2.3 Expression of *cry*1*Ac* gene

A white band appeared around 130 kD by SDS-PAGE, in agreement with the result expected (Fig. 3).

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ataatgggcg agaagtaagt agattgttaa caccctgggt caaaaattga tatttagtaa   60
aattagttgc actttgtgca tttttcata agatgagtca tatgttttaa attgtagtaa   120
                          -35box                   -10box
tgaaaaacag tattatatca taatgaattg gtatcttaat aaaagagatg gaggtaactt   180
atggataaca atccgaacac caatgaatgc attccttata attgtttaag taaccctgaa   240
ORF ──►
gtagaagtat taggtggaga aagaatagaa actggttaca ccccaatcga tatttccttg   300
………………………………………………………………………………………………………………
………………………………………………………………………………………………………………
……………………………………………………………… gaattactcc ttatggagga atagtctcat   3720
                                                   End codon
gcaaactcag gtta                                                     3734
```

Fig. 3 The partial sequences of *cry*1*Ac*20 gene

2.4 Bioassay of *cry*1*Ac* gene products

Second or 3rd instar larvae of *Pieris rapae* L. had been used for bioassay. Bioassay results (Table 3) showed that translated products was high toxic to *Pieris rapae* L., the mortality of larvae was 88.89% 48 h after their feeding with leaves immersed in cultures containing transformants, and the growth of one or two larvae survived was seriously inhibited. While the control has little influence on the growth of larvae and the mortality was only 1.11%. These results indicated that *cry*1*Ac*20 gene cloned in this study has high insecticidal activity against Lepidoptera insects and consequently applicable potential.

Table 3 Bioassay results of engineered DHF10 to *Pieris rapae* (after 48 h)

Samples	Dilution	Repeat	Number of larvae tested	Number of larvae died	Mortality (%)	Corrected mortality (%)
DHF10	1%	3	90	80	88.89	88.78
	10%	3	90	75	83.33	83.33
CK	1%	3	90	1	1.11	–
	10%	3	90	0	0	–

3 DISCUSSION

Most of the *cry*1*Ac* genes cloned were originated from *B. thuringiensis* subsp. *kurstakai*. The *cry*1*Ac* gene cloned in this study was originated from strain Rpp02 which belongs to *Bacillus thuringiensis* subsp. *morrisoni* and was isolated from Sichuan ecological condition, suggesting Bt resource has distinctive feature under Sichuan ecology. This further broadened the resources of bacteria with *cry*1*Ac* gene. The cloning of *cry*1*Ac*20 gene (GenBank accession number DQ285666) not only gave birth to a new member of *cry* gene family, but also provided a good gene resource for construction of transgenic plants using novel transgenic engineering bacteria.

The *cry*1*Ac*20 gene shares a stunning 99% homology in nucleotides sequence with that of known *cry*1*Ac* family, except a few changes of amino acids. It was reported that toxic polypeptides of *cry*1-type insecticidal crystal proteins have 3 Domains. Mutation of Domain Ⅰ affects the binding ability to receptors (Wu and Aronson., 1992); mutation of Domain Ⅱ affects toxicity without affecting the binding ability to receptors (Smedley and Ellar, 1996); and mutation of a conservative segment of Domain Ⅲ affects toxicity and the formation of spore channels (Chen *et al*., 1993). The changing place of amino acids encoded by *cry*1*Ac*20 gene, compared with other *cry*1*Ac* genes, mainly occurs at toxic fragment of N-terminals, maybe in Domain Ⅰ, domain Ⅱ, or Domain Ⅲ, or even the whole fragment. As a result, even subtle changes of gene sequences can lead to different toxicities. In addition, some changes of amino acids from hydrophobic to hydrophilic do have effect on the sheeting of proteins, which affects protein 's structures and functions in return.

In the work of studying *cryIA*(*b*) of Bt aizawai HD133, Chak and Jen (1993) found that 72 nucleic acid sequences upstream could manipulate expression of the gene in *E. coli*; accordingly, they thought these sequences may probably harbor initiator sequence able to be expressed in *E. coli*. The forward primer P1 was located on 180 bp upstream of initial condon. Thereby the PCR products encompass not only coding areas of *cry*1*Ac* but also its own initiator and upstream areas of the initiator that is important in the process of transcription. Results in this study revealed this gene could be expressed in *E. coli*, and its expressed product was highly toxic to *P. rapae* larvae, further

proving pre-expectations and precious reports by Kin *et al*. that nucleic acids sequences upstream of ORF could exhibit feature of initiator and were able to manipulate gene expression. The cloning of *cry*1*Ac*20 gene provided a good genetic material for constructing high efficiency insecticidal engineering microbial. Exorbitant expression of genes may be possible if the optimal nutritional ingredients proportion of engineering bacteria was determined and provided.

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（责任编辑：袁德成）

苏云金芽孢杆菌 Rpp02 菌株 *cry*1*Ac*20 基因的克隆及表达

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摘要：Rpp02 **菌株是本实验室分离的一株对鳞翅目等多种害虫具有高毒力的苏云金芽孢杆菌莫里逊亚种**（*Bacillus thuringiensis* subsp. *morrisoni*）**，经** PCR **检测，它含有** *cry*1*Ac* **基因。对其基因组** DNA **进行** PCR **扩增，得到大约** 4 kb **的产物。测序结果表明，该片段含有一个较大的** ORF **框，基因编码区为** 3 534 bp**，编码** 1 177 **个氨基酸，分子量为** 133.144 kD，pI 4.952**，为弱酸性蛋白质，亮氨酸**（Leu）**、丝氨酸**（Ser）**、谷氨酸**（Glu）3 **种氨基酸含量最高，分别为** 8.0%**、**7.8%**、**7.7%**。该基因序列与** *cry*1*Ac* **序列同源性达到** 99%**，并被国际** Bt **杀虫晶体蛋白基因命名委员会命名为** *cry*1*Ac*20**。生物测定表明，该基因在大肠杆菌中得到了表达，表达产物具有较强的杀虫效果，试喂菜青虫** 48 h **后，校正死亡率为** 88.78%**。**

关键词：苏云金芽孢杆菌；_cry_1_Ac_20 **基因；克隆；表达；菜青虫**

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